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14. ABSTRACT

Stem cells (SCs) are present in specialized niches within tissues and enable long-term organ function by replacing dying cells over time. SCs have the unique capability for self-renewal and can be stimulated to transform into different cell types within the organ. Since SCs are genetically coded to live longer than other cells and resist mechanisms that would normally induce cell death, they may be especially susceptible to carcinogenic changes. Recent evidence suggests that cancer stem cells (CSCs) exist and are responsible for cancer propagation, despite representing the minority of cells within tumors. Consequently, the development of drugs that target CSCs may result in better clinical outcomes. As a first step, identifying CSCs in solid tumors must occur. Early studies to identify SCs within normal prostate and prostate cancers are promising, but limited by the difficulty of cultivating these rare cells. Development of the prostasphere assay, using cells derived from human tissue specimens, may enable in-depth characterization of prostate stem cells. Prostaspheres are globes of stem and early progenitor cells that can be induced to grow in culture. The prostasphere assay may enable enrichment of SCs from normal and cancerous specimens for further characterization, with the long-term goal of defining new therapeutic targets within this population.

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Table of Contents

INTRODUCTION:

The overall objective of this proposal was to identify a prostate cancer stem cell population that may be targeted in an effort to eradicate disease that is not effectively managed by localized treatments. To do this, we generated a collection of human prostaspheres, derived from benign and malignant surgical specimens. Studies in other organ systems have indicated that sphere-forming cells are predominately stem and early progenitor cells. Therefore, the sphere assay may be utilized to expand and cultivate the rare prostate epithelial stem cell population. Characterization of prostaspheres could then exploited to provide a wealth of information related to normal prostate development as well as tumor biology. Biomarker identification on putative stem/progenitor cells may lead to novel therapeutic targets. We formulated an approach for studying the stem cell properties of cells comprising prostaspheres, outlined in the specific aims.

BODY:

As described above, several studies in multiple organ systems have shown that cells with stem-like qualities can form spheres. These sphere-forming cells can be induced to differentiate in vitro, or form glands and recapitulate tumors via reconstitution experiments in vivo in a variety of organ systems. The development of prostasphere technology may enable in-depth characterization of stem cells derived from normal and cancerous prostate tissue, with the long-term translational goal of exploiting specific therapeutic targets within this population. As a first step in this developmental project, we proposed the following exploratory aims.

Aim 1: Develop Conditions for Human Prostasphere Formation
Aim 2: Assess Stem-Cell Properties of Human Prostaspheres

Tasks Involved in Accomplishing Aim 1:
1) Acquisition of human prostate tissue specimens from patients undergoing prostate surgery
2) Determine appropriate culture conditions (i.e., tissue dissociation, media, additives) for prostapshere formation

Materials/Methods/Outcomes (Aim 1): (Please see Appendix for manuscript with figures)

Patient Population and Tissue Acquisition: We recruited patients undergoing prostate surgery at UCLA to participate in tissue donation to the UCLA Epithelial Tumor Bank via an approved protocol through the Office for the Protection of Research Subjects (OPRS). Informed consent was obtained from willing subjects. Once the surgical specimen was removed en bloc, it was brought to the surgical pathology grossing room, where a pathologist assisted in obtaining samples of prostate tissue in a sterile manner. An adjacent section from each specimen was paraffin-embedded for histological determination of the presence of normal or cancerous glands. The fresh prostate tissue samples were placed on ice, followed by immediate transport to the laboratory for processing. All surgical specimens were coded for patient anonymity.

Tissue Dissociation and Culture: Prostate tissue was weighed and then minced into small pieces via sharp dissection. Tissue fragments were digested in a 0.25% solution of type I collagenase overnight. After 12 hrs, cells were washed in phosphobuffered saline (PBS), followed by further digestion with trypsin. Dissociated cells were separated from undigested organoids by sequential filtering through 100- and 40-micron mesh. Single cell suspensions were divided and mixed in suspension with 50:50 Matrigel:prostate epithelial cell basal medium (PrEGM, Clonetics®), or as a monolayer of prostate epithelial cells on adherent plates. In all of our experiments, cells were counted and assessed for viability after dissociation and prior to plating and/or passaging. Quantitation of prostaspheres derived from each specimen was performed. Aliquots of dissociated cells/spheres were isolated and maintained in liquid nitrogen storage for future analysis.

Development of Human Prostaspheres: We generated spheres from prostate tissue specimens of 60 patients undergoing cancer surgery. Prostaspheres developed indiscriminately from all tissue types, regardless of Gleason grade, with 1 to 4% of unfractionated cells capable of sphere formation. Variability in the efficiency of

4

prostasphere development was observed among samples but did not appear to be associated with any patient clinical or pathological characteristics.

Prostaspheres Express Basal Markers: The prostate epithelium is composed of basal cells, including stem cells and transient amplifying cells, terminally differentiated luminal cells, and neuroendocrine cells. Prostate epithelial cells can be differentiated based on expression of a variety of markers. The basal markers CK5, alpha 6 integrin (CD49f), CD44, and p63 were strongly expressed by the majority of sphere-forming cells. On the other hand, luminal markers, including AR (androgen receptor) and PSA (prostate-specific antigen) were not observed in prostaspheres cultured in the absence of androgen. CK8, a marker expressed in intermediate and luminal cells, was occasionally noted in sphere-forming cells. In these CK8+ cells, co-expression with CK5 was also demonstrated. Expression of the neuroendocrine marker, synaptophysin was not observed. Taken together, the pattern of expression of sphere-forming cells most closely resembles normal basal cells; with predominate CK5, CD44, alpha integrin (CD49f), and p63 expression.

Tasks Involved in Accomplishing Aim 2:
   1) Evaluate self-renewal capability of prostaspheres
   2) Evaluate differentiation/tissue regeneration potential of prostaspheres

Materials/Methods/Outcomes (Aim 2):
To assess the self-renewal potential of human prostaspheres, dissociation of the spheres into single cell suspensions were performed followed by re-plating in Matrigel. New spheres were observed in independent experiments with a variety of patient specimens for more than 16 generations. Prostaspheres that were culture for >2 weeks in Matrigel formed branching structures, demonstrating differentiation capability. Please see appendix for manuscript with all figures.

Tissue Regeneration Capability of Prostaspheres:
In addition to in vivo branching structures, subcutaneous injection of prostaspheres combined with Matrigel and rat urogenital sinus mesenchyme (UGSM) into immunocompromised mice resulted in xenograft formation with acinar-like structures resembling partially differentiated prostate glands. These glands demonstrated expression of several prostate markers, including prostate stem cell antigen (PSCA), AR, CK5, CK8, and p63.

KEY RESEARCH ACCOMPLISHMENTS:

Determination of in vitro culture condition for development and expansion of human prostaspheres
Demonstration of self-renewal capability of prostapsheres
Antigenic profile of sphere-forming cells
Demonstration of branching morphagenesis of sphere-forming cells in vitro
Demonstration of tissue-regeneration capability of prostaspheres in vivo

REPORTABLE OUTCOMES: We have generated a manuscript of our findings that we are in the process of submitting. This manuscript is included in the appendix for a detailed description of all materials, methods, and findings related to prostaspheres.

CONCLUSIONS:

Since initiation of funding, we have accomplished all of the goals outlined in the original statement of work. Additionally, we have generated an extensive collection of human prostaspheres from the spectrum of prostate tissue specimens that are viable in culture and are a continuous source of human material for the study of prostate gland development and tumor initiation in vitro and in vivo. The foundation of work enabled by this grant will enable future funding to continue investigating prostate stem cells via this approach.

APPENDICES: Manuscript entitled "Sphere-forming cells derived from prostate tumors demonstrate stem/early progenitor traits, but do not contain the TMPRSS-ERG fusion". Figure slides 1-6.

Sphere-forming cells derived from human prostate tumors demonstrate stem/early progenitor traits, but do not contain the TMPRSS-ERG fusion

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Abstract:

We generated prostaspheres from tumor specimens of 59 patients undergoing cancer surgery. Collected prostate tissue included the full spectrum of normal and cancerous (Gleason grade 3-5) prostate glands. Selected tumors were assessed for the *TMPRSS-ERG* gene fusion by fluorescence activated in situ hybridization (FISH). Human prostaspheres developed from all tissue types with 0.5% to 4% of unfractionated cells capable of sphere formation. Variability in prostasphere quantity was not associated with any patient clinical or pathological characteristics. Sphere-forming cells could remain quiescent for weeks in culture until the addition of growth factor-supplemented media induced the development of prostaspheres. These prostaspheres could be dissociated in bulk and passaged long-term (>16 generations). Cell sorting revealed that prostate cells expressing CD44 and CD49f formed prostaspheres. Immunostaining of prostaspheres displayed a predominantly basal cellular phenotype (CD44+CD49f+CK5+p63+CK8-AR-PSA-). Prolonged in vitro three-dimensional (3D) culture and/or exposure to androgen resulted in branching of prostaspheres with the emergence of more mature markers (CK8 and AR) in a subset of sphere-forming cells. Subcutaneous injection of prostaspheres combined with urogenital sinus mesenchyme in immunocompromised mice resulted in the development of acinar structures. Surprisingly, the *TMPRSS-ERG* fusion was not detected in sphere-forming cells obtained from fusion-positive tissues. Our findings of quiescence, self-renewal, and differentiation of prostaspheres indicate that this may be a functional approach for characterization of prostate stem/early progenitor cells. The absence of *TMPRSS-ERG* fusion indicates that either these cells may not contain gene rearrangements, or that prostate cancer stem cells containing the *TMPRSS-ERG* fusion have distinct requirements for survival and expansion in vitro.

Introduction:

Adult multipotential stem cells (SCs) are responsible for the development, maintenance, and regeneration of the range of specialized cell types comprising mammalian tissues[1, 2]. Self-renewal is a fundamental characteristic of SCs and refers to asymmetric divisions that give rise to genetically identical daughter cells in addition to more differentiated progenitors. Substantial evidence suggests that this process may be deregulated in cancer, as transformed SCs or early progenitors demonstrate uncontrolled self-renewal that results in phenotypically diverse tumors [3-5]. Isolation of SCs may allow the mechanisms that regulate their activity to be delineated. Acquiring phenotypic identity as well as a functional understanding of SCs could lead to new therapeutic approaches for cancer[5, 6].

SCs should demonstrate self-renewal, quiescence, high proliferation capacity under appropriate stimulation, and multi-lineage differentiation[1, 3]. Approaches used to identify human prostate SCs include functional evaluation of fractionated cells expressing SC markers identified in other organ systems, in vivo regeneration capacity of fractionated cells, and characterization of side populations [7-10]. Richardson et al found that the $\alpha_2\beta_1^{hi}$-integrin cells co-expressing CD133 had increased proliferative activity and appeared to regenerate acinar structures when combined with stroma in immunocompromised mice[10]. CD133+/ $\alpha_2\beta_1^{hi}$ cells have been identified in numerous studies and consistently represent a small fraction (<1%) of prostate epithelial cells in benign and cancer tissues[8, 10-12]. The marker profile of normal and cancer SCs may coincide, since CD44+CD133+$\alpha_2\beta_1^{hi}$ cells from prostate tumors also displayed increased proliferative capacity and multi-lineage differentiation in vitro[11].

HTeRT-immortalized human prostate epithelial cells acquired from high-grade (Gleason 8-10) tumor specimens were evaluated by Gu et al. [9]. Highly proliferative clones were tested for regenerative activity in vivo and xenografts were obtained that recapitulated the primary tumor's glandular structure. These clones expressed embryonic SC markers (Oct-4, nanog) as well as the putative prostate SC markers CD44 and CD133. The accumulation of recent work on human prostate stem cells suggests that a small proportion of basal cells expressing integrin, CD44, and CD133 may delineate this population in normal prostate tissue and cancer. However, further studies inclusive of a

3

broad range of benign and cancerous human prostate cell types are still needed to confirm a marker profile that encompasses the functional activities definitive of SCs.

In order to adequately analyze human prostate SCs from a variety of tissue types, methods that enable the efficient isolation and expansion of these rare cells is critical. Implementing culture conditions that support proliferation of cells that form spheres may enrich for SCs [8, 13-16]. Spheres are multicellular globes that develop from cells that survive anchorage-independent conditions in vitro, such as growth in ultra-low attachment (ULA) plates or 3D Matrigel cultures. Spheres include SCs and early progenitors in studies of breast, brain, and skin and are frequently used to study the processes of self-renewal and differentiation in these systems [13, 17-19]. Human sphere formation from dissociated prostate tissue is also observed [8-10, 13, 20, 21]. Lang et al demonstrated spheroid formation when primary human prostate cells were grown in 3D Matrigel cultures[22]. Branching morphogenesis with expression of luminal markers in response to androgen and stromal growth factors was also noted, suggesting the versatility of this approach for studying differentiation cues, in addition to profiles of anchorage-independent cells. Murine prostate studies have also demonstrated Sc characteristics contained within the sphere-forming population of cells. In these studies, 0.1 to 0.5% of dissociated prostate cells formed clonally derived prostaspheres in Matrigel cultures[13]. Murine prostaspheres developed exclusively from Sca-1+ cells expressing high levels of alpha 6 integrin (CD49f) and demonstrated self-renewal and branching morphogenesis with luminal differentiation in vitro and in vivo[13, 16]. Because sphere-forming capacity appears to be a property of SCs, some human studies have used prostasphere formation as a functional validation of prostate SC activity in fractionated cell lines or xenografts[9, 23, 24].

Although it clear that human prostaspheres develop and undergo branching morphogenesis, the characteristics of quiescence, self-renewal, and clonality of sphere-forming cells have not been thoroughly examined. It is also unclear whether sphere-forming cell derived from tumors represent cancer SCs. In the present study, we have accrued a diverse collection of human prostate tumors and found that prostaspheres form reproducibly and robustly from any prostate tissue type. In the absence of appropriate growth factors, sphere-forming cells remain dormant for weeks in culture. Once

established, however, clonally derived prostaspheres can be dissociated and passaged for multiple generations. In concurrence with previous human spheroid studies, branching morphogenesis is observed in vitro and in vivo with the formation of ductal/acinar-like structures. Alpha 6-integrin (CD49f) and CD44 are markers that enrich the sphere-forming population, with approximately 1 out of 15 cells capable of forming spheres. The quiescent, self-renewing, and differentiation features of prostaspheres provide support for the preservation of SC activity in these cultures. Surprisingly, prostaspheres generated from tumor specimens containing the *TMPRSS-ERG* fusion failed to demonstrate this gene rearrangement. This result suggests the possibility that prostate cancer stem/progenitor cells do not contain gene rearrangements. Alternatively, prostate cancer SCs containing the *TMPRSS-ERG* fusion may have requirements for survival and expansion in vitro that are lacking in our current culture conditions.

Materials and Methods:

Tissue acquisition, isolation and culture of prostate epithelial cells:
Human prostate tissue was obtained from 59 patients (ages 41-76), undergoing retropubic prostatectomy for adenocarcinoma of the prostate or cystoprostatectomy for bladder cancer. All subjects were consented for tissue collection in accordance with an approved protocol through the office for the protection of research subjects at UCLA. For a list of patient characteristics, see Table 1. Adjacent tissue specimens were fixed in formalin and paraffin-embedded to determine the presence of benign or malignant glands. The remainder of the tissue specimens were placed on ice and brought immediately to the laboratory for mechanical and enzymatic digestion as previously described. Briefly, prostate tissue was sharply minced into small fragments ($1mm^3$) in RPMI supplemented with 10% FBS. Tissue fragments were washed once and incubated for 12 hours in 0.25% type I collagenase (5ml/gram). Organoids were washed in RPMI and treated with trypsin (TripLE, Invitrogen) for 5 minutes at 37^0C. Dissociated tissue cellular suspensions were sequentially filtered through 100-micron and 40-micron filters. After filtration, cell suspensions were passed through a 23-gauge needle. Cells were counted with a hemocytometer and resuspended in PREGM (Clonetics) supplemented with FGF2

(Invitrogen), EGF (Sigma), B27 (Invitrogen), and heparin (Sigma). The cells were then seeded in ULA plates (Corning), matrigel (BD), or subjected to cell separation using MACS beads columns (Miltenyi Biotec LTD, Surrey, UK), as described below.

Antigenic cell separation:

Cells were subjected to the MACS microbeads linked to a cocktail of lineage antibodies (Lineage Depletion Kit, Miltenyi Biotec Ltd, Surrey, UK). After selection through the magnetic column, lineage negative cells were incubated with anti-CD44, anti-CD49f, or anti-CD133 antibodies, followed by incubation with MACS goat anti-mouse IgG microbeads (Miltenyi) and application to a MACS column. Alternatively, Lin- Cells stained with fluorescent-linked primary antibody were subjected to cell sorting. Antigen enriched or depleted cells were counted and resuspended in defined media prior to plating in 3D Matrigel cultures.

In vitro prostasphere culture:

Epithelial cells were counted and re-suspended in 50:50 matrigel:PREGM a concentration of $1x10^3$-$6x10^4$ cells/80microliters. This matrigel/cellular suspension was plated at the edge of the well on 12-well plates and allowed to set by incubation at 37^0C for 30 minutes. One milliliter of defined media was then added to each well and plates were replaced in 37^0C incubator. For dissociation and passage of prostaspheres, media was removed and replaced with fresh media containing 1mg/ml Dispase (Invitrogen) and incubated at 37^0C for 1 hour. Spheres were colleted, washed in RPMI, and the incubated with TripLE (100microliters/12-well plate) for 5 minutes at 37^0C. Five volumes of PREGM were added for washing and cells were counted and replated as described above.

Lentiviral Infection of prostate epithelial cells:

Primary prostate tissue was mechanically and enzymatically dissociated as described above. Single prostate epithelial cells were cultured in PREGM for 48-72 hours. The media was removed and viral supernatant containing lentivirus (CCR-dsRed, a gift from the laboratory of Dr. Irvin Chen at UCLA) and polybrene was added for 3 hours at 37^0C. The cells were then washed and fresh PREGM added. Red fluorescence was detected in

cells 48-72 hours post-infection. Monolayer cells were detached with TripLE and plated in matrigel as described above. Spheres were noted to develop 4-10 days after plating in matrigel.

Immunohistochemistry of tissue/prostasphere sections:
Prostate tissue was paraffin embedded as previously described. For paraffin embedding of prostaspheres, matrigel cultures were subjected to Dispase (1mg/ml, Invitrogen) and whole prostasphere were collected and washed. Prostaspheres were fixed in 10% buffered formalin at 4^0C for twelve hours. After fixation, prostaspheres were washed in PBS, followed by 50% ethanol. Prostaspheres were pelleted by centrifugation and resuspended in 10-20microliters of Histogel (Richard-Allen Scientific). The Histogel containing the prostaspheres was then subjected to frozen section or paraffin embedding. Four-micron thick sections of frozen or paraffin embedded tissue were deparaffinized with xylene and rehydrated through a descending series of ethanol washes as described. Antigen retrieval and standard immunoperoxidase procedures were used in combination with primary antibodies. For Immunofluorescence assays, permeablization of tissue was performed using cold Methanol:acetone, followed by staining with primary antibodies against the antigen of interest, then secondary antibodies containing the immunofluorescent marker.

Fluorescence-activated cell sorting and analysis:
Prostate cells were suspended in PBS/10% FCS and stained with antibody for 30 minutes at 4^0C. FACS was performed using the FACScanner and CellQuest software. Cell sorting was done using the FACS Vantage or Aria sorters (BD Biosciences).

Subcutaneous Injections in Immunocompromised mice:
Male SCID mice age 8-24 weeks were subjected to subcutaneous injections of prostaspheres +/- 2 x 10^5 rat urogenital sinus mesenchyme (UGSM) suspended in 100 microliters 50:50 matrigel:PREGM. Mice injected with spheres/UGSM combination also received subcutaneous implantatl of testosterone pellets for time-release supplementatl testosterone. Mice were monitored for 8-12 weeks and then sacrificed. Subcutaneous

nodules at the site of injection were dissected and frozen and paraffin-embedded sections were prepared for immunohistochemical analysis. Rat UGSM was prepared as previously described[25].

Fluorescence Activated In Situ Hybridization:

Paraffin-embedded human prostate tumor specimens were subjected to FISH as previously described[26].

Results:

A small fraction human prostate cells obtained from a diverse collection of human prostate tumor specimens form prostaspheres

To investigate whether sphere-forming cells may be derived from all types of human prostate tissue, specimens were obtained from 59 patients undergoing radical prostatectomy or cystoprostatectomy. Pathological examination of collected specimens confirmed inclusion of either benign (normal and BPH) or a mixture of benign and malignant glands with Gleason grades ranging from 3-5 (Table 1). Fresh tissues were mechanically and enzymatically dissociated into single cell suspensions as described in the Materials and Methods. Single cells were counted followed by seeding in ULA plates or in Matrigel at densities ranging from 10 to 1×10^5 cells/well. Of 21 patient specimens cultured in ULA plates and 35 specimens cultured in Matrigel, all formed prostaspheres (data not shown). Culturing prostaspheres in Matrigel became our preferred culture method because it prevents aggregation and facilitates enumeration, dissociation, and passage [13, 27]. Prostasphere formation was noted within 3-5 days of plating, with continued growth over 2 weeks to diameters of 100-400 microns (Figure 1). Although significant variability in the number of prostaspheres that developed from individual patient specimens was observed, consistent variation in the number or appearance of prostaspheres according to any clinical or pathological parameters was not apparent (Figure 1A-F). Tallies of prostaspheres were obtained from 10 individual patients with varying pathologies (benign to malignant) after replicate plating in 6-well culture dishes. The average number of prostaspheres obtained with similar seeding densities per patient

was plotted (Figure 1G). The frequency of sphere-forming cells was found to range from approximately 0.5%-4%.

The development of prostaspheres depended on the presence of permissive culture conditions, specifically requiring supplemented PREGM media for efficient growth. PREGM is a defined media that is frequently used to establish and maintain primary prostate cultures in vitro[28]. As described in the Materials and Methods, PREGM is supplemented with a variety of growth factors that enable maximal sphere growth [15]. Unsupplemented PREGM supports prostasphere development at a reduced growth rate (data not shown). On the other hand, prostaspheres did not form if cells were cultured in WAJC 404, another defined media that is used to establish prostate epithelial monolayer cultures [29, 30]. Dissociated prostate cells were plated in triplicates of 10^4 cells/well in WAJC 404 or PREGM media (Figure 2). As expected, prostaspheres were noted by 7 days in cultures containing PREGM (Figure 2A). WAJC 404 cultures were observed for 4 weeks with fresh media added weekly, but no prostaspheres developed during this period (Figure 2B,C). When WAJC 404 media was removed after 4 weeks and replaced with PREGM, abundant prostasphere growth was noted within 7 days (Figure 2D). In contrast, matched 4-week old cultures that received fresh WAJC never developed prostaspheres (data not shown). Delayed prostasphere formation suggests that sphere-forming cells resist apoptosis in environments that do not support growth by remaining quiescent until stimulatory conditions are obtained.

Human prostaspheres are clonally derived and self-renew

To confirm the clonal origin of prostaspheres, 10^5 freshly isolated prostate epithelial cells were grown as a monolayer for 48 hours and then labeled with red fluorescent protein via lentiviral-mediated gene transfer of the dsRed gene. Approximately 90% of prostate cells appeared to be expressing dsRed within seventy-two hours after exposure to virus (data not shown). Infected (red) prostate epithelial cells were detached and mixed with wild-type (colorless) cells at a ratio of 1:4. The mixed cell populations were plated in Matrigel in triplicate. Approximately 7 days following plating, robust prostasphere formation was observed and only monochromatic (all red or

9

all colorless) prostaspheres were identified, consistent with the concept of clonality (Figure 2E).

Of the initial prostasphere specimens cultured in Matrigel, 12/14 could be passaged for multiple generations. Repetitive passaging was specifically assessed in 4 individual prostasphere cultures and more than 16 generations were obtained without any sign of growth decline after dissociation and passage (data not shown). DsRed-infected prostaspheres could also be dissociated, mixed, and passaged repeatedly (>3 generations) with formation of new monochromatic prostaspheres. Single red fluorescent prostaspheres were isolated by serial dilution in 96-well plates, followed by dissociation and incubation of single cells in Matrigel. Secondary red prostaspheres were noted to develop, supporting the self-renewal potential of individual prostaspheres (Figure 2F-G)

Prostate cells obtained from dissociated prostaspheres also remained viable after freeze/thaw, with formation of new spheres in Matrigel that could be serially passaged. Dissociated prostaspheres (passage 6-10) were cryopreserved for 1 month followed by thaw and seeding in Matrigel. New prostaspheres developed 7-10 days after plating that could be passaged >3 generations (data not shown). Taken together, these findings highlight the versatility of prostaspheres, in that they can be genetically manipulated, expanded continuously in vitro, and cryopreserved.

Prostaspheres express predominately basal markers

The prostate epithelium is composed of basal cells, including stem cells and transient amplifying cells, terminally differentiated luminal cells, and neuroendocrine cells[31]. Prostate epithelial cells can be differentiated based on expression of a variety of markers[32]. Basal cells express the high molecular weight cytokeratins, CK5 and CK14, as well as low levels of AR, correlating with their relative androgen insensitivity. Basal cells also express p63, CD44 and alpha integrin[33]. Previous studies of human prostate stem cells have suggested that basal cells expressing CD44, high levels of $\alpha^2\beta^{1Hi}$ integrin, and CD133 mark this population. Luminal cells, in contrast, do not express p63, but exhibit high levels of AR, PSA, and the low molecular weight cytokeratins, CK8 and CK18[32]. Transient amplifying (intermediate) cells express basal marker, CK5 and often co-express the luminal marker, CK8. Neuroendocrine cells are typically identified

based on their expression of neuropeptides, such as chromogranin A and synaptophysin[32].

In order to determine the expression profile of prostate epithelial cells that compose prostaspheres, immunostaining was performed on frozen and paraffin-embedded prostaspheres using antibodies against several basal and luminal markers (Figure 3). Prostaspheres isolated from Matrigel after 2 weeks of culture appear as solid clusters of cells (Figure 3A). Upon H&E staining of sectioned prostaspheres, the architecture of sphere is delineated. The typical appearance is two fairly uniform layers of cells surrounding an inner cellular mass (Figure 3B). Some of the prostaspheres appear to contain secretions at the center, and this phenomenon is more evident with prolonged culture in Matrigel as discussed below.

The basal markers CK5, alpha 6 integrin (CD49f), CD44, and p63 were strongly expressed by the majority of sphere-forming cells. Luminal markers, including AR (androgen receptor) and PSA (prostate-specific antigen) were not observed in prostaspheres. The lack of AR and PSA expression is not surprising since these spheres were cultured in the absence of serum and androgen, which may prevent expression of detectable levels of AR and hormone-responsive genes, like PSA. CK8, another marker expressed in intermediate and luminal cells was occasionally noted in sphere-forming cells. Co-expression with CK5 was seen in these cells (Figure 3G-H). We did not observe any staining for the neuroendocrine marker, synaptophysin (data not shown). Taken together, the pattern of expression of sphere-forming cells most closely resembles basal cells; with predominate CK5, CD44, alpha integrin (CD49f), and p63 expression.

In vitro differentiation of prostaspheres

Previous studies of human prostate spheroids have indicated that they can differentiate in matrigel to form ductal/acinar structures that express luminal markers, when exposed to androgen or stromal conditioned media[10]. We observed branching of prostaspheres in vitro if cultured for approximately 30 days in Matrigel. These complex branching structures often appeared highly organized (Figure 3K-L). When sphere-forming cells were cultured in the presence of 10^{-7} M testosterone, branching was appeared more abundant with regression of spheroid structures (data not shown).

Immunostaining of the branched prostaspheres demonstrated a predominantly basal phenotype (Figure 3O-R). However, a larger subset of cells from these cultures appeared to express the luminal marker CK8 compared to the 2-week old cultures (Figure 3P). Additionally, the concomitant loss of p63 was often seen in CK8-positive cells, suggesting differentiation to a more luminal phenotype. Immunostaining of AR or PSA in branching prostaspheres was only detected in a few cells cultured in the presence of androgen (Figure 3Q-R). Our findings indicate that branching morphogenesis occurs in prostaspheres after prolonged culture in Matrigel with induction of AR and PSA expression observed in rare cells if cultured with androgen.

Antigenic profile of human prostasphere-forming cells

To determine what cell surface markers could be used to identify cells capable of prostasphere formation, we performed a series of experiments upon subpopulations of prostate cells fractionated by microbead or automatic cell sorter separation (Figure 4). By subjecting cells from dissociated tissue to fractionation experiments, we observed a variable decrease (2-10 fold) in the overall number of prostaspheres formed following manipulation (data not shown). However, by comparing fractionated cell populations from individual specimens subjected to similar treatments, relative sphere-forming activity was reliably and reproducibly obtained.

In order to eliminate the possibility that cells of the hematopoeitic lineage retained in the prostate tissue contributed to the sphere-forming population, we performed depletions with blood lineage antibodies. FACS analysis with representative lineage antibodies CD31 (data not shown) and CD45 (Figure 4B) confirmed removal of hematopoeitic cells. Lin- and Lin+ cells were plated in Matrigel cultures in several replicates of 1×10^4 cells/well to assess sphere-forming activity. The Lin+ cell cultures did not form prostaspheres, with all of the sphere-forming activity evident within the Lin- cell fraction (Figure 4C).

Previous investigations of human and murine prostate stem cells have suggested that CD44 may be a marker expressed by this population[10]. In order to investigate whether Lin- prostate cells that express CD44 were enriched for sphere-forming ability, we used microbeads or automatic cell sorting to isolate cells expressing this antigen.

FACS analysis confirmed enrichment of CD44+ cells (Figure 4B). Plating of CD44+ cells in Matrigel resulted in enriched for sphere-forming capability >2.5-fold with 1/15 cell forming spheres in the Lin-CD44+ population (Figure 4C). Although the presence of CD44 greatly enriched for sphere-forming capability, some spheres were noted in the Lin-CD44- fraction. This could represent incomplete separation of CD44+ and CD44- cells. On the other hand, it is possible that a fraction of Lin-CD44- cells may have sphere-forming capability.

We used a similar approach of antigenic separation to examine whether CD49f and the putative human prostate stem cell marker, CD133, demonstrated increased sphere-forming capability in sorted cell populations. FACS analysis demonstrated a small population of CD49f+ (8-12%) and CD133+ cells (2-7%) after lineage depletion (data not shown). Fractionation with MACS beads enabled enriched and depleted populations to be isolated as confirmed by FACS (Figure 4B). Analysis of fractionated cells in 3D Matrigel cultures revealed marked increase in sphere-forming ability in Lin-CD49f+ compared to Lin-CD49f- (Figure 4C). Similar to what was seen with CD44, a small fraction of CD49f cells were capable of forming spheres. Multiple attempts to separate CD133+ and CD133- cells resulted in highly variable results in sphere-forming capability. For most of the patient samples, more spheres appeared to form in the CD133- fraction, however, a smaller number of spheres consistently formed within the CD133+ fraction as well (Figure 4C). In one case, the CD133-enriched fraction selected for higher sphere-forming capability (data not shown). Our difficulties in obtaining consistent results with CD133 cell separation could be due to the fact that the CD133+ cells are so rare, usually composing 0.25-2% of unfractionated prostate cells. This made isolation and plating in spheres cultures difficult. We typically isolated <2x10^3 CD133+ cells via this approach.

Prostaspheres do not contain the *TMPRSS-ERG* gene rearrangement

Since the human prostaspheres were generated from primary tumors, we presumed that in vitro cultures would include clonally derived benign and cancerous spheres, reflective of the heterogeneity of glands found in tissue specimens. We could not distinguish prostaspheres derived from cancerous or benign specimens based on

phenotype, marker expression, or growth rate. With the discovery of prevalent gene rearrangements involving ETS family members in prostate cancer, we anticipated that cytogenetic tools may enable identification of cancerous prostaspheres[34]. Gene fusions involving *ERG*, *ETV1*, and *ETV4* involve a variety of 5' partners that direct aberrant expression of these transcription factors and possibly initiate a cascade of events leading to tumorigenesis [34, 35]. The most common rearrangement involves juxtaposition of the androgen-regulated *TMPRSS2* gene with *ERG*. *TMPRSS-ERG* gene fusions have been detected in primary prostate tumor specimens, metastases, and xenografts by fluorescence in situ hybridization (FISH)[26]. Analysis of prostate tumor surgical cohorts have found 36-78% of prostate cancers possess the *TMPRSS-ERG* fusion[35]. We wondered whether we could use *TMPRSS-ERG* to distinguish normal and malignant prostaspheres. The presence of this fusion in individual prostaspheres may suggest that cancer stem/early progenitor cells can be expanded in our cultures.

To test the feasibility of this approach, FISH analysis was performed on select prostate tissue specimens and coordinating prostaspheres (Figure 5). The *TMPRSS-ERG* fusion was found in approximately 60% of cancer cases tested. Surprisingly, the fusion was conspicuously absent from prostasphere cultures derived from *TMPRSS-ERG+* tissues, even when the specimens contained >90% tumor (Figure 5B). Analysis of monolayer cultures concomitantly derived from prostate tumor specimens also failed to demonstrate the presence of the gene fusion, indicating that both spheroid and adherent cultures select for fusion-negative cells (data not shown).

Sphere-mediated prostate tissue regeneration:

To evaluate whether human prostaspheres can form ductal/acini structures in vivo, whole prostaspheres from two different patients were injected subcutaneously into SCID mice with or without $2x10^5$ rat UGSM cells. The number of prostaspheres injected ranged from $4 x10^2$ to $1 x 10^6$, with 4/8 mice injected with spheres alone forming grafts and 2/4 mice injected with spheres + UGSM forming grafts by 8 weeks post injection (Figure 6A). The size of the grafts correlated with the number of spheres injected (data not shown). Control mice injected with rat UGSM only or the combination of rat UGSM and UGSE, were included for comparison (Figure 6A-E). Grafts were fixed in formalin

and embedded in paraffin to prepare tissue sections. H&E staining revealed the prostaspheres regenerated acinar-like structures, however branching ducts were not observed in the spheres only and spheres + rat UGSM grafts (Figure 6D-E). No acini were observed in the UGSM only grafts (Figure 6B) and typical 1-2 cell layered rodent prostate ductal/acinar structures were noted when rat UGSM was combined with rat UGSE. Grafts generated from prostaspheres in the absence of UGSM demonstrated rudimentary appearing acini with multiple layers of epithelial cells and little lumen formation (Figure 6D). In the setting of combining human prostaspheres with rat UGSM, acini contained well-defined lumens (Figure 6E). IHC analysis showed that these acinar structures were reminiscent of normal adult human adult prostate glands with CK8 and AR positive luminal cells and p63 positive basal cells (Figure 6H-M). CK5, although usually confined to the basal layer in human prostate glands, was observed throughout the full thickness of the epithelium in the regenerated glands (Figure 6N-O). PSA was not detected in any of the acinar structures (Figure 6T-U), although staining for the prostate-specific markers prostate stem cell antigen (PSCA), and prostate membrane antigen (PSMA) was observed (Figure 6P-S).

Although it appears that prostaspheres can regenerate xenografts resembling normal human prostate, the efficiency of glandular structure formation was relatively low and the glands were not completely differentiated, as evidenced by lack of PSA staining and only a small subset of cells expressing CK8. Our results indicate that a subset of prostaspheres is capable of glandular regeneration, albeit at a low efficiency and without the full compliment of ductal/acinar structures and marker representation observed.

Discussion:

Sub-populations of cancer cells that have the ability to initiate and propagate tumors share many similarities to tissue stem cells and are often referred to as cancer stem cells (CSCs) [6]. The possibility of targeting CSCs with the goal of obtaining more definitive cancer therapy has resulted in efforts to identify this highly specialized populations from normal prostate as well as adenocarcinoma in mice and humans [9-11, 16]. Sphere-formation in anchorage-independent conditions is a well-established characteristic of SCs in mammary and neural systems and a common approach for their

15

isolation [15, 17]. Studies of murine prostate have demonstrated that a small fraction of prostate epithelial cells expressing Sca-1 and alpha-6 integrin (CD49f) formed spheres in 3D-Matrigel cultures that possessed self-renewal and differentiation characteristics [13, 16, 22, 36]. In human studies, prostate spheroid formation with branching morphogenesis and marker differentiation in vitro has been observed, but self-renewal capability and antigenic profiling was not addressed [36, 37]. Here, we show that 0.5%-4% of epithelial cells obtained from a wide variety of dissociated human prostate specimens form prostaspheres that possess features of self-renewal, in addition to branching morphogenesis. Human prostaspheres can be dissociated, passaged, and cryopreserved with the retention of sphere-forming ability following thaw. These practical traits of human prostaspheres may enable viable repositories of prostate stem/progenitor cells to be generated that could facilitate high throughput studies of large collections of patient specimens in the future.

In prior human prostate stem cell studies, epithelial cells expressing $\alpha_2\beta_1^{hi}$ integrin, CD44, and CD133 have displayed stem-like qualities of increased proliferative potential in vitro and in vivo regeneration of acinar-like structures [7, 10, 11]. Cells were isolated based on rapid adhesion to collagen and demonstrated bright alpha-2 integrin (CD49b) expression. Although the expression of other integrins was not thoroughly addressed, it was noted that cells expressing alpha-6 integrin (CD49f) were contained within the isolated cells [7]. Interestingly, some studies of CD49f in prostate cancer have indicated that its expression may be preserved in cancer cells, while many of the other integrins, including CD49b, are lost [38]. Consistent with previous reports characterizing prostate SCs, the putative SC markers, CD44 and CD49f appeared to greatly enhance for the sphere-forming population in our current study. The CD133+ population was technically difficult to evaluate, given the small fraction of these cells present in human prostate tissue specimens. Expression of CD133 did not appear to be a distinct and consistent advantage of for prostasphere formation, since prostaspheres were observed in both positive and negative fractions, with more spheres consistently seen in the CD133-isolates. Additionally, immunostaining of whole prostaspheres failed to detect CD133 expression and FACS analysis/RT PCR of dissociated prostaspheres did not detect CD133, indicating that this marker is not preserved in prostasphere cultures or required

16

for continued passage (data not shown). On the other hand, CD44 and CD49f, were retained in the sphere cultures throughout passaging (see Figure 3). Although a small number of spheres were observed in the CD44- and CD49f- fractions, most of the activity was found in the positive fractions with a sphere forming rate of 1/15 plated cells. The presence of spheres in the CD44- and CD49- fraction may represent a population of luminal cells that have self-renewal capability and we are currently in the process of attempting to enrich for these cells for more extensive characterization.

The basal marker profile of sphere-forming cells with abundant expression of CK5 and p63 may suggest that normal cells may be selected in our prostasphere cultures. Basal markers are frequently lost in cancer tissues with malignant glands displaying a more luminal phenotype that includes abundant CK8/18 expression, variable AR, and loss of CK5 and p63[39]. The acinar structures that we observe *in vivo* also appeared to resemble normal human prostate tissue with abundant basal marker expression. Although many of the collected tissue specimens contained large tumor volumes (>90% tumor glands), all of the prostaspheres derived from the spectrum of prostate tissue samples demonstrated a predominance of basal cells. It has been a consistent challenge in the field to distinguish primary prostate epithelial cell cultures derived from normal and cancerous tissue specimens and to confirm that cells derived from cancer specimens, in fact, represent malignant cells[40]. The lack of markers that clearly delineate normal from cancerous prostate epithelial cells contribute to this dilemma. With the discovery of the ETS family fusions that are detectable via FISH, we were able to take advantage of a new method to determine whether genetic events associated with malignancy are preserved in prostaspheres[34, 41]. Strikingly, although we were able to generate prostaspheres from prostate tumor specimens demonstrating the *TMPRSS-ERG* fusion, this translocation was not preserved in the spheres. In this study, *TMPRSS-ERG* cells could not be detected in monolayer cultures or sphere cultures generated from fusion-positive tumors. This result raises the possibility that prostate cancer cells have a distinct growth disadvantage compared to normal cells under the culture conditions utilized here. With the application of new surface markers that may allow separation of cancer and normal prostate epithelial cells, this issue can be further addressed and conditions that may allow expansion of prostate cancer spheres may be interrogated.

Based on previous studies in other organ systems, spheres can exhibit full differentiation potential, in addition to self-renewal capability[17, 18, 42]. In the case of mammospheres, induction of functional lactating glands is possible[19]. In the current study, we observed luminal markers typically found in terminally differentiated prostate cells only in a small minority of cells composing branching prostaspheres in cultures exposed to androgen. Additionally, our xenografts generated in vivo by combining the prostaspheres with rat UGSM yielded rudimentary acinar, without evidence of ductal structures and incomplete maturity of luminal cells, demonstrated by lack of PSA expression. One possibility for the limited differentiation potential in vivo and in vitro is that a significant proportion of prostaspheres develop from progenitor cells, not bona fide SCs. To distinguish between human prostate SCs and progenitors, it will be necessary to assess the ability of fractions of sphere-forming cells to regenerate glandular structures in vitro and in vivo. It is possible that bona fide SCs will be able to fully recapitulate normal/cancer prostate glands, while the progenitors will demonstrate more restricted capacity for differentiation and glandular regeneration. Further antigenic profiling and correlation with glandular/tumor regeneration capability is needed to determine the proportion of SCs versus progenitors within our prostasphere-forming population.

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Tables

Patient Code	Age	Race	PSA	CA Locus	Grade	pStage
R060106	64	Asian	8	R,L,A,P	4+4	T2cNxMx
R060606	45	caucasian	4.8	RA,LB,LA	3+3	T2cNxMx
R061606	49	caucasian	NA	R	3+3	T2cNxMx
R062606	61	caucasian	NA	L	3+3	T2aNxMx
R071006	58	caucasian	NA	RA, RL.LL	3+3	T2cNxMx
R072406A	60	caucasian	NA	RA, RL	3+3	T3bNxMx
R072406B	69	hispanic	NA	RA, RL,LL	3+4	T2cNxMx
R072506	41	African-american	NA	R/L	3+4	T2cNxMx
R073106A	51	caucasian	3.5	R/L, A/P	3+3	T2cNxMx
R073106B	47	caucasian	NA	R/L,A/P	3+4	T2cN0Mx
R060106	64	Asian	8	R,L,A,P	4+4	T2cNxMx
D080406	69	caucasian	4.25	R/L, A/P	3+3	T2cN0MX
L081406	54	hispanic	NA	R/L	3+3	T2cN0MX
D082106	59	caucasian	3	LA	3+3	T2aN0Mx
R082606	51	African-american	NA	LA	3+3	T2aN0Mx
R091106	51	caucasian	2.75	R,L,A	3+3	T2cN0Mx
L091106	60	hispanic	19	R,L,A, A/P	3+4	T2cN0Mx
B100306	64	caucasian	6	R,L,A/P	3+3	T2cNoMx
D100606	67	caucasian	NA	Negative	Benign	NA
R100906	69	caucasian	NA	R,L,A/P	3+4	T2cNxMx
B100906	61	middle eastern	5	R,L,A/P	2+3	T2cN0Mx
L100906	60	caucasian	2.4	R,L	3+3	T2cN0Mx
D101606	66	caucasian	NA	L	3+4	T2aN0Mx
D101706	50	caucasian	5.41	R,L,A,P	3+3	T2cNxMx
S102306	64	caucasian	NA	Negative	Benign	NA
R103006	57	caucasian	NA	R	3+4	T2bNxMx
R103106	76	caucasian	NA	R,L,A/P,A	3+4	T2cnxMx
D110106	50	caucasian	75	R,L,A/P,A	4+3+5	T3aN0Mx
R110606	58	caucasian	NA	R,L,A/P,A	4+3+5	T2cNXMX
B011707	64	caucasian	4.3	R/L,A/P,B	3+3	T2cN0Mx
L030207	59	caucasian	5	R/L,A/P,B	3+4	T2cN0Mx
L030507	62	caucasian	5.4	R/L,A/P,	4+5	T3aN0Mx
S042307	58	caucasian	NA	R,L,A,P	4+3	T2cN0Mx
S050207	69	armenian	13	R,L,A,P	4+3	T3aN0Mx
D050207	70	caucasian	9.4	R,L,A,P	3+4	T2cN0Mx
R061807	61	caucasian	4.3	R,L	3+3	T3aNxMx
D080607	60	african-american	11	R	3+3	T2aN0Mx
R081307	62	caucasian	4.7	R,L	3+4	T2cNxMx
R091707	60	caucasian	3.9	R,L,A,P	3+3	T2cN0Mx
D092407	58	african-american	2.8	R,L	3+3	T2cN0Mx
R100107	67	caucasian	NA	R,L,A,P, Ap	3+3	T2cN0Mx

D100107	66	Asian	6.7	R	3+3	T2bN0Mx
R100807	51	caucasian	5.7	R,L, Ap	3+4	T2cN0Mx
D100807	76	caucasian	9	R,L,Mid,A	3+3	T2cN0MX
R102207	57	caucasian	4.6	R	3+3	T2aN0Mx
D111507	53	caucasian	4.1	R, P, Ap	3+3	T2aN0Mx
JR111907	73	caucasian	5	L, R, A, P	3+4	T2cN0Mx
R111907	69	caucasian	6.4	L,R,A,P	4+3	T2cNxMx
S120307	51	caucasian	8.5	R, A	3+4	T2aNxMx
S120907	54	caucasian	5.2	L,R,A,P	3+4	T2cNxMx
D121007	57	caucasian	1.8	L,R,A,P	4+5	T3aNoMx
R010708A	53	hispanic	9.5	L,P,Ap	3+4	T2cN0Mx
R010708B	51	caucasian	7.2	L,R,A,P,Ap	3+4	T2cNxMx
R011408	58	Asian	3.3	L,R,P,Ap	3+4	T2cNxMx
S011508	68	Asian	5.4	L,R,A,P,Ap	4+3	T2cNxMx
D012808A	58	caucasian	7.4	R,L,P	3+4	T2cNoMx
R020408	51	Hispanic	4.5	R,L,A,P	4+3	T2cNxMx
B021108	65	caucasian	5	R,L,A,P	3+4	T2cNoMx

Figure/Table Legends:

Table 1: Patients undergoing radical prostatectomy for prostate cancer were consented for tissue donation according to an approved protocol through the Office for the Protection of Research Subjects at UCLA. Patient information including age, pre-operative prostate specific antigen (PSA), tumor location on final pathology (R, Right, L, Left, A, Anterior, P, Posterior, Ap, Apex, B, Base), and pathological stage (T2, tumor confined within the prostatic capsule, T3, tumor extends beyond the prostate capsule) are shown in the table.

Figure 1: Formation of Prostaspheres from Dissociated Human Prostate Tissue. Schematic diagram of prostate specimen processing (top). Samples of human prostate tissue obtained from patients undergoing cancer surgery were mechanically and enzymatically digested. Single cells were and plated in Matrigel seeded at a density $2x10^4$/well in 6-well culture dishes. Adjacent tissue samples were paraffin-embedded and stained with H&E for histological evaluation. Human prostaspheres developed

robustly from all prostate specimens. A. Benign prostate tissue, B. Specimen containing Gleason 3+3 adenocarcinoma. C. Gleason 4+5 adenocarcinoma. Prostaspheres derived from these tissues formed prostaspheres in Matrigel (D-F). There were no observable differences in the prostasphere size, shape or architecture among the various samples. Additionally, there was no significant difference in sphere-forming capability in cells derived from benign or cancerous tissue. G. Average prostasphere counts/seeded cells from 7 individual patients with pathological diagnosis of benign prostate, low- grade, and high-grade adenocarcinoma.

Figure 2: Prostaspheres remain quiescent in non-permissive media. Dissociated human prostate cells were plated in 50:50 matrigel:WJAC 404 media and incubated for one month. No prostaspheres were observed under these media conditions (A), although robust sphere formation was observed with supplemented PREGM media (data not shown). WJAC 404 media was removed after four weeks in culture and replaced with supplemented PREGM. Prostaspheres formed within one week after the addition of the permissive media (B), indicating sphere-forming cells remain in a dormant state in non-permissive growth conditions. Evaluation of clonal origin and self-renewal of prostaspheres. Freshly dissociated prostate cells were grown as a monolayer for 48 hours followed by incubation with lentivirus carrying the gene for red fluorescent protein (RFP). Red cells were mixed with uninfected (colorless) cells at a ratio of 1:4 and plated in Matrigel culture (1×10^4 cells/well). Only monochromatic spheres were observed in (E) as seen in phase contrast and dark fields views. Monochromatic spheres were isolated and plated by serial dilution into ULA 96-well plates so that 1-sphere/well was obtained. Single spheres were subjected to digest with TrypLE and single cells were then re-

incubated in PREGM media (F). New monochromatic spheres were noted to form from single cells after 2-weeks of incubation (G).

Figure 3: Human prostaspheres exhibit a predominately basal expression profile. Paraffin-embedded human prostate tissue and prostaspheres were stained for prostate epithelial markers. A. Matrigel culture of prostaspheres. B. H&E staining of paraffin-embedded prostaspheres. C. Human prostate stained for CK8 (red), and p63 (blue). D. Human prostate tissue stained for CD44 (green) with DAPI nuclear staining (blue). E. Human prostate staining for CK5 (green) and CD49f (blue). F. Human prostaspheres stained for CK5 (green) and DAPI (blue). G. Human prostaspheres stained for CK8 (red) and p63 (blue). H. Merged field of human prostasphere staining for CK5 (green), CK8 (red), and p63 (blue). I. Human prostasphere stained for CD44 (green) with DAPI nuclear staining (blue). J. Human prostaspheres stained for CD49f (green) with DAPI nuclear staining (blue). K. Paraffin-embedded human prostaspheres after long-term culture in Matrigel resulted in branching morphogenesis. L. High power (20x) view of branching structures. M, N. H&E staining of paraffin-embedded branching prostaspheres. O. Branching prostaspheres stained with CK5 (green) and DAPI (blue). P. Branching prostaspheres stained with CK8 (red) and p63 (blue). Q. Branching prostaspheres stained with PSA (red) and p63 (blue). R. Branching prostaspheres cultured with 10-7mM testosterone and stained with antibodies against androgen receptor (AR).

Figure 4: Antigenic Marker Separation and Evaluation of Sphere-forming capability. Lin-CD44+ cells are enriched for sphere-forming capability. A. Schematic diagram of cell fractionation based on surface marker expression using magnetic beads or automated cell sorting. B. FACS analysis of dissociated prostate cells subjected to enrichment/depletion of lineage, CD44, CD49f, or CD133 antigens. C. Graphs representing the average number of spheres found per well of isolated cells after antigenic separation of dissociated prostate tissue. At least 3 different patient samples
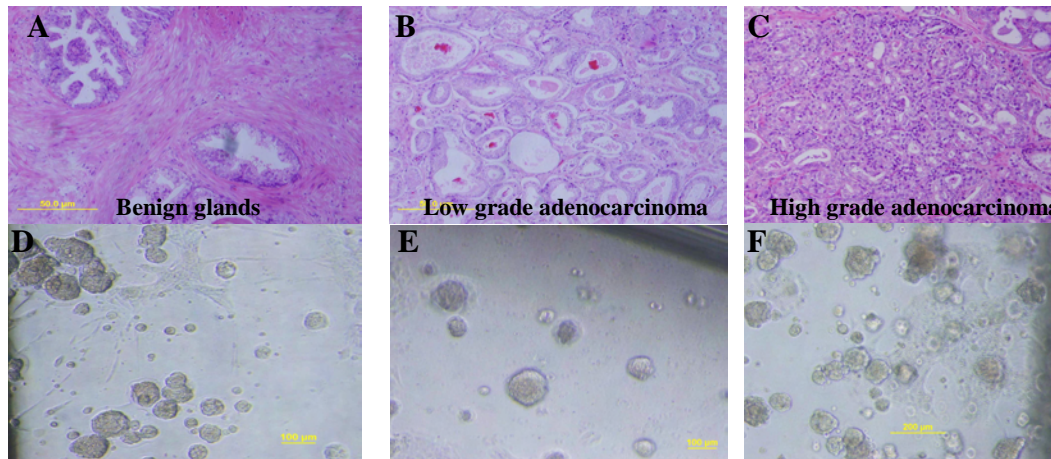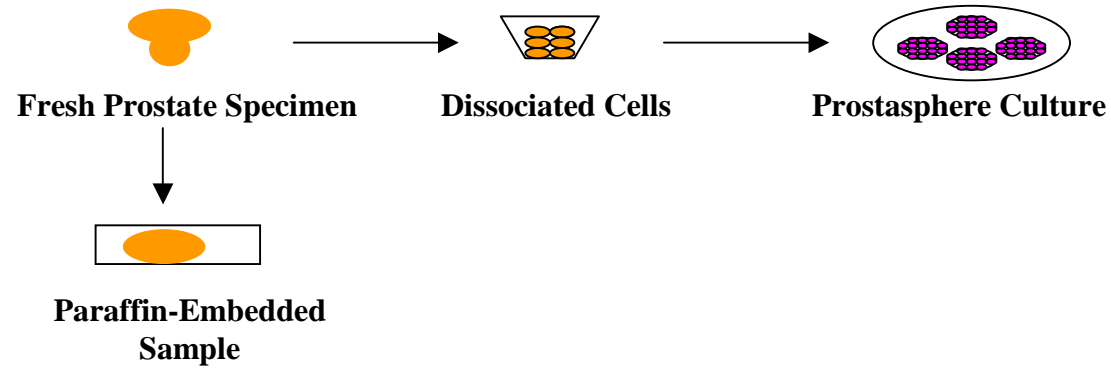
were tested per antigen with consistency of sphere-forming capability among enriched/depleted fractions. Lineage, CD44, and CD49f isolates were plated at a rate of 1×10^4 cells per well. CD133 isolates were plated at 1×10^3 cells/well

Figure 5: Patients undergoing radical prostatectomy for prostate cancer were consented for tissue donation according to an approved protocol through the Office for the Protection of Research Subjects at UCLA. Patient information including age, pre-operative prostate specific antigen (PSA), tumor location on final pathology (R, Right, L, Left, A, Anterior, P, Posterior, Ap, Apex, B, Base), and pathological stage (T2, tumor confined within the prostatic capsule, T3, tumor extends beyond the prostate capsule) is presented in the panel A. Tissue specimens from these patients were mechanically and enzymatically dissociated and cultured in 50:50 Matrigel:PREGM (supplemented with EGF, FGF, and B27) to allow prostasphere formation. Paraffin-embedded tumor specimens and prostaspheres were subjected to FISH for *TMPRSS:ERG* gene rearrangement. A single prostasphere stained with cytokeratin 5 (green) and DAPI (blue) is shown adjacent to a FISH image of a single sphere (B). None of the prostaspheres demonstrated *TMPRSS:ERG* fusion.
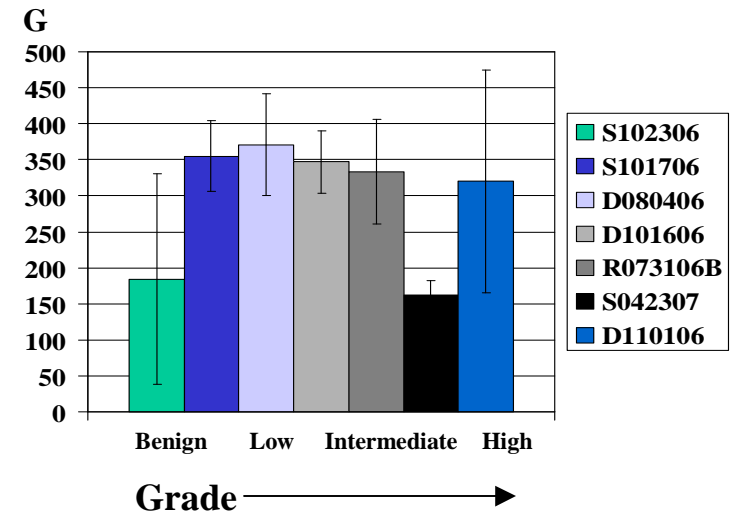
Figure 6: Prostaspheres form acinar structures in immunocompromised mice. Whole prostaspheres were isolated, mixed with Matrigel and injected subcutaneously into mice either with or without rat UGSM. Glandular structures could be palpated 6-12 weeks post-injection. A. Glandular-like structures recovered 8 weeks after subcutaneous implantation of approximately 1×10^5 whole prostaspheres suspended in Matrigel. B. H&E staining of graft containing rat UGSM only. C. H&E staining of the graft containing rat UGSM combined with rat UGSE to demonstrate the appearance of regenerated rodent glands. D. H&E stain of regenerated prostate tissue obtained from grafts containing whole prostaspheres without UGSM, showing tightly packed acinar structures separated by mouse infiltrating stroma. E. H&E stain of regenerated prostate tissue formed by combining 1×10^4 whole prostaspheres with 2×10^5 rat UGSM. F,G: High magnification of regenerated human glands from prostaspheres mixed with rat UGSM. H-

U. IHC analysis of the expression of AR, p63, CK8, CK5, PSCA, PSMA, and PSA in normal human prostate tissue sections (control) and acini derived induced by prostaspheres (graft).
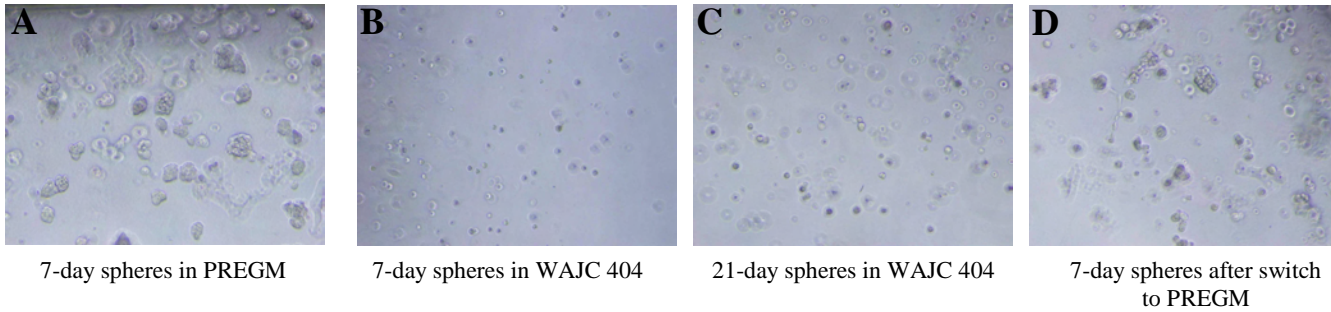
Prostaspheres form from dissociated prostate tissue regardless of clinical or pathological features
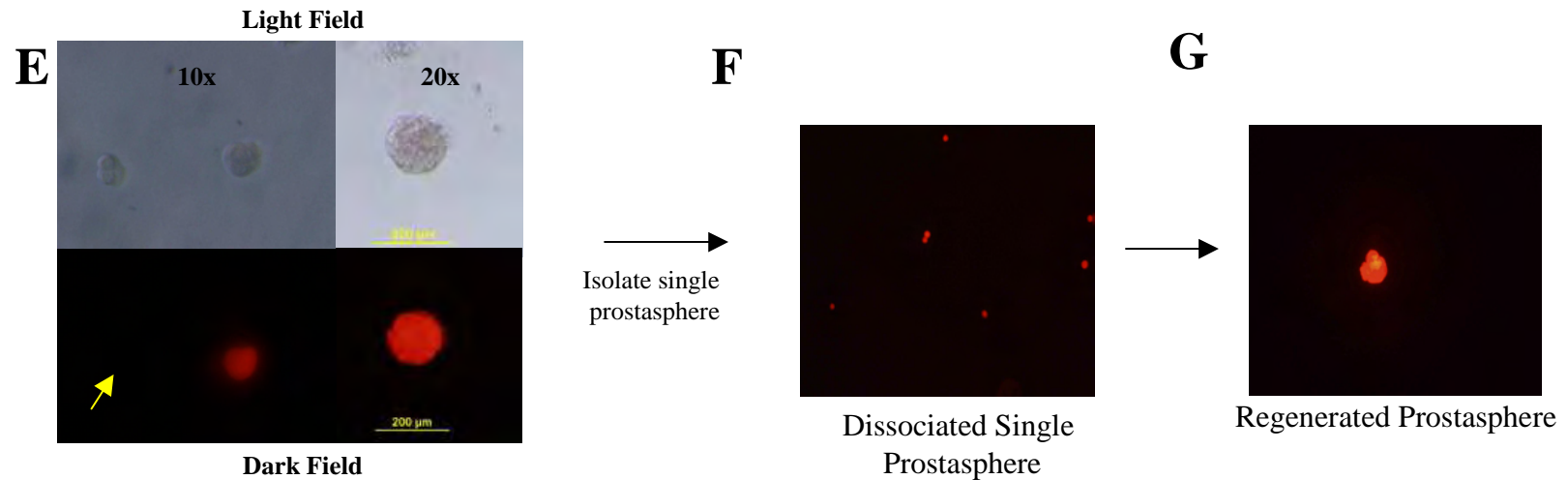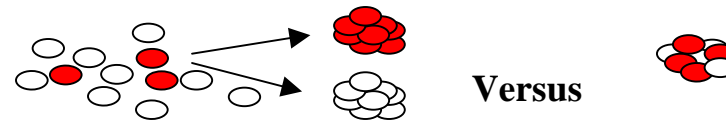

Fresh Prostate Specimen → Dissociated Cells → Prostasphere Culture
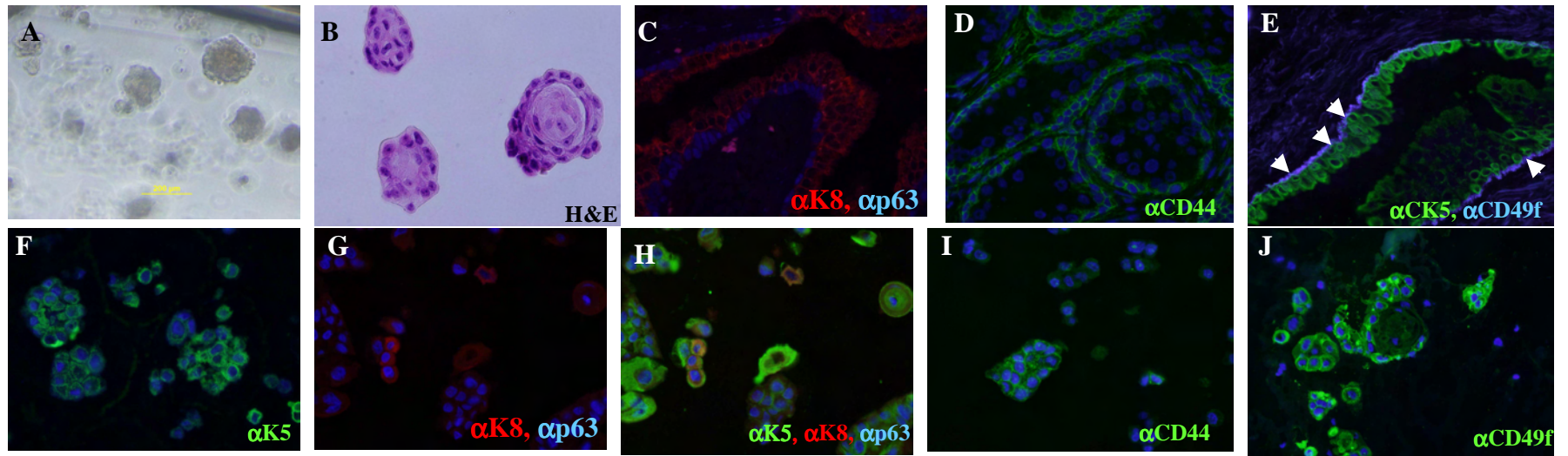
Paraffin-Embedded Sample

A — Benign glands

B — Low grade adenocarcinoma

C — High grade adenocarcinoma

D

E

F

Prostasphere derived from tumor specimens

G

Grade →

S102306
S101706
D080406
D101606
R073106B
S042307
D110106

Sphere-forming cells remain dormant but viable

A — 7-day spheres in PREGM

B — 7-day spheres in WAJC 404

C — 21-day spheres in WAJC 404

D — 7-day spheres after switch to PREGM

Evaluation of clonal origin and self-renewal of prostaspheres

Versus

Light Field

E
10x
20x

200 µm

Dark Field

F

Isolate single prostasphere

Dissociated Single Prostasphere

G

Regenerated Prostasphere

Human prostaspheres express basal markers

Prostaspheres form branching structures in vitro
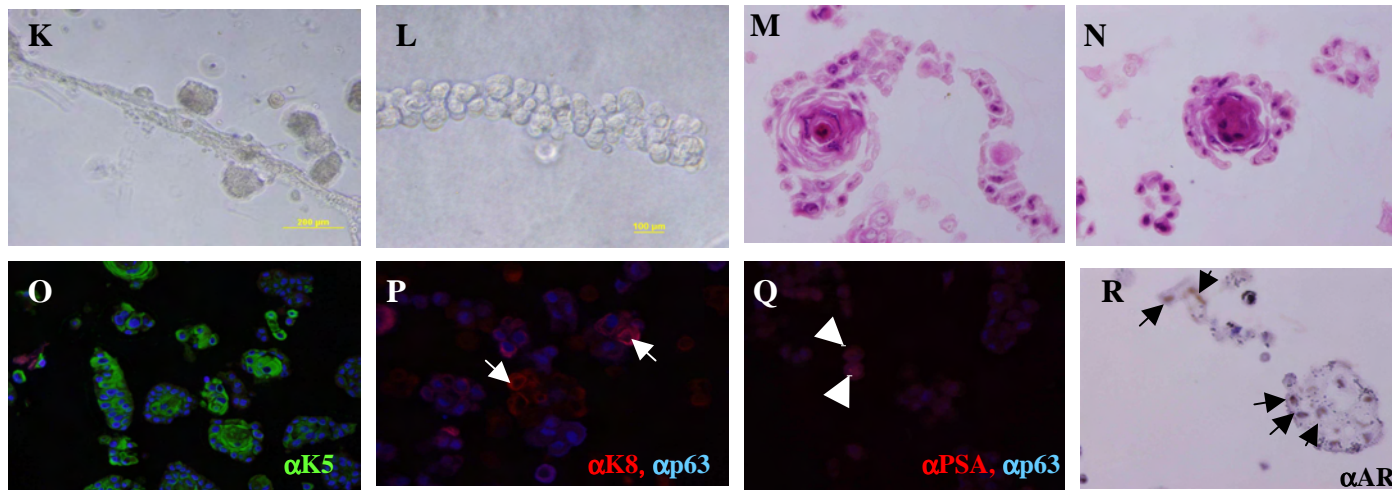
A

Antigenic marker separation of prostate cells

Prostate tissue

↓

Dissociated cells

↓

Lineage Depletion
MACS beads column

↓ → FACS/Culture Lin+ and Lin- cells

Primary antibody (CD44, CD49f, or CD133)
MACS beads IgG column or cell sorting

↓

FACS/Culture enriched and depleted fractions
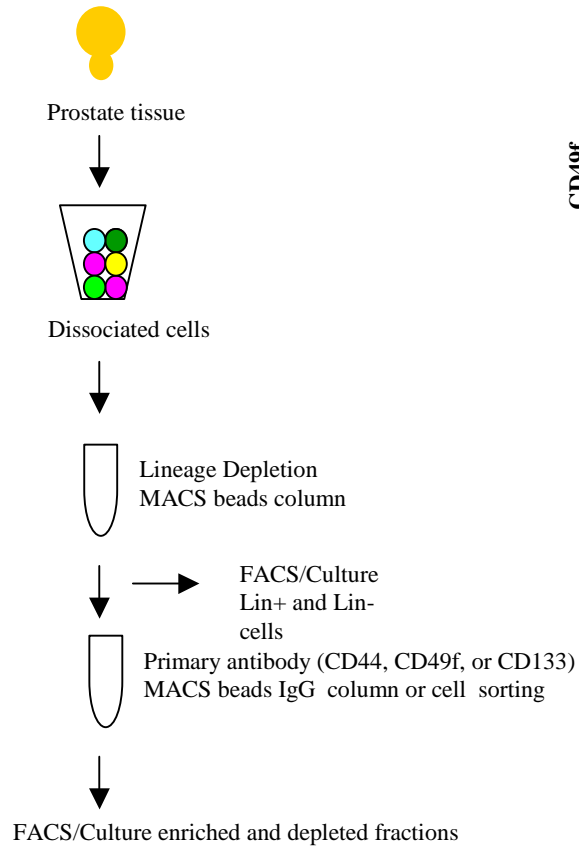
B   FACS confirms enrichment/depletion based on antigenic markers
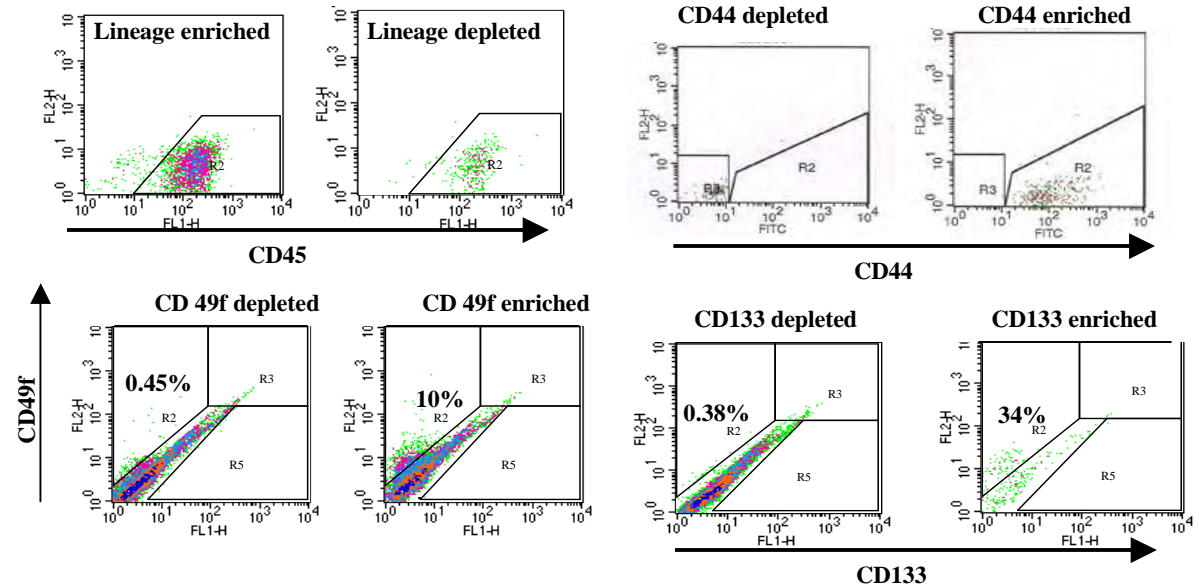
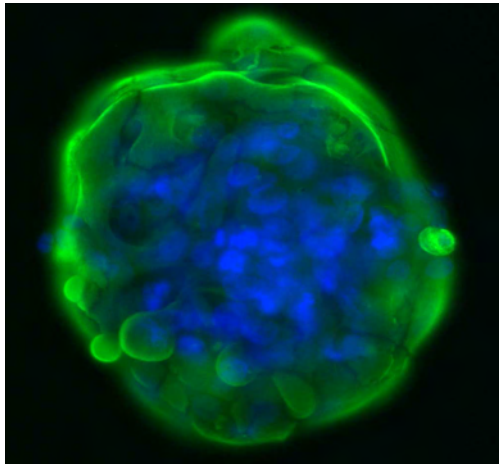## C   Prostate cells expressing CD44 and CD49f demonstrate majority of Sphere-forming activity
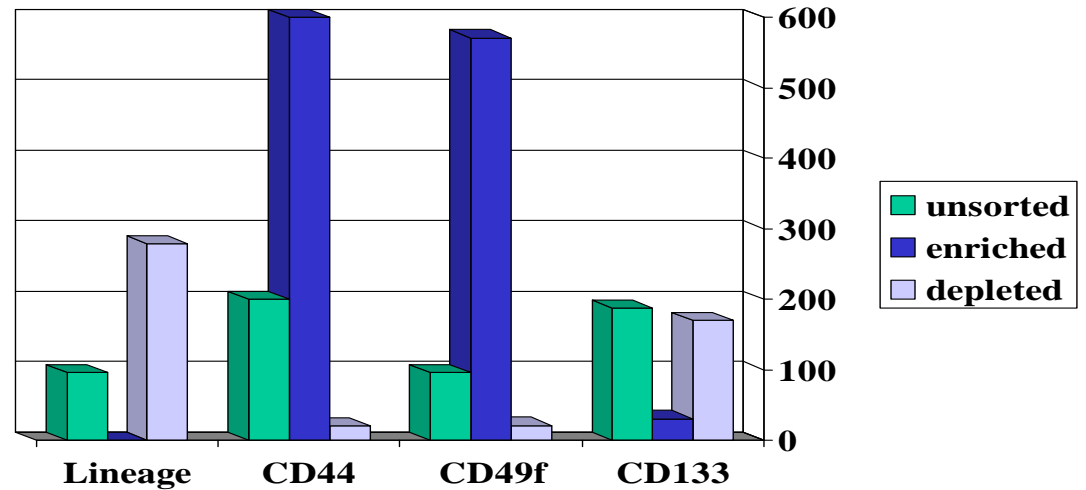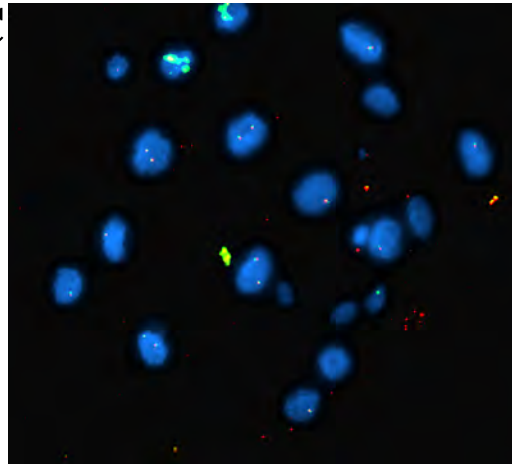
Prostaspheres do not contain TMPRSS-ERG fusion

A

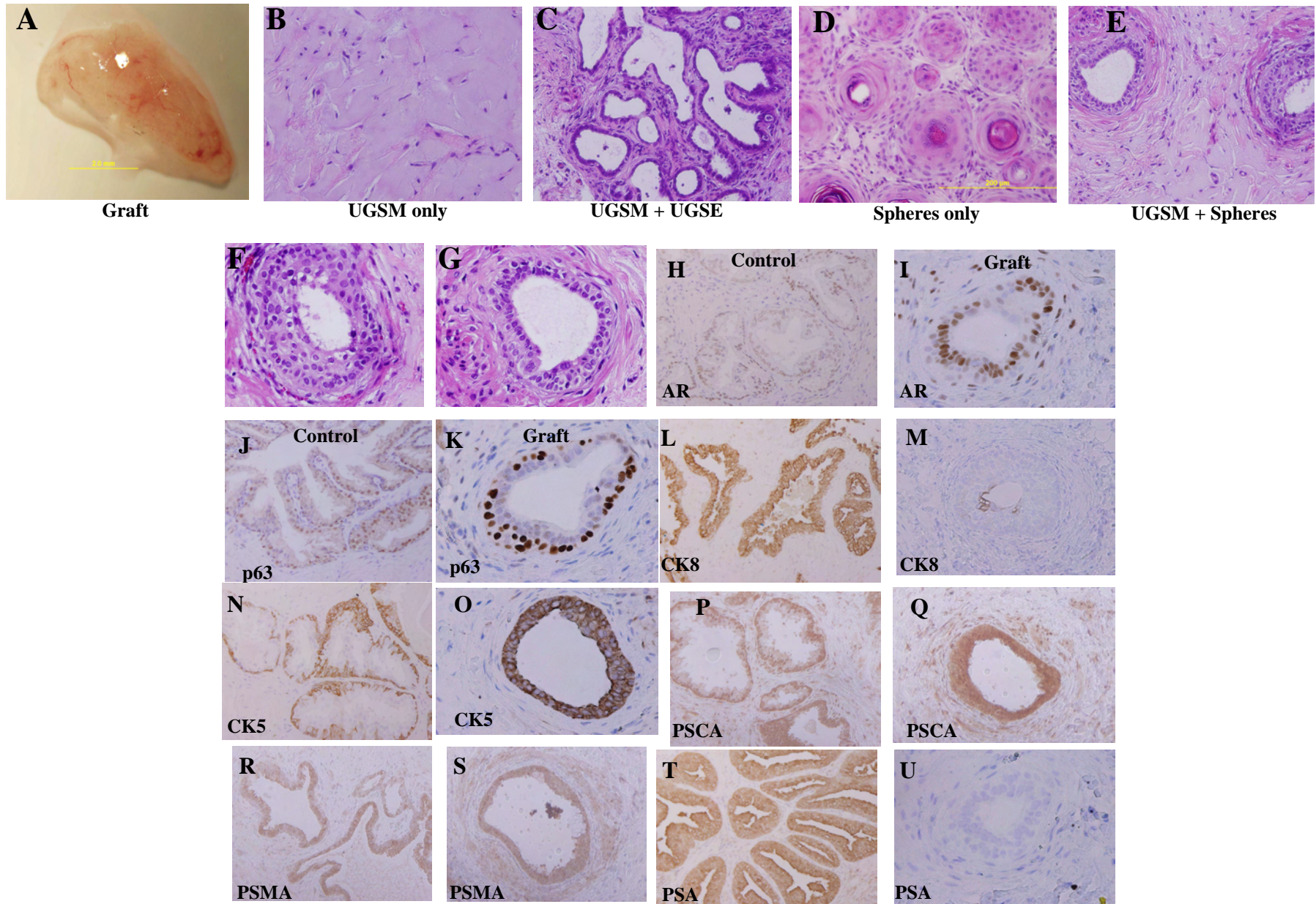| Patient Code	Age	CA Locus	Grade	pStage	TMPRSS2:ERG	FISH of Spheres
R072406A	60	RA, RL	3+3	T3bNxMx	Positive	Negative
R073106A	51	R/L, A/P	3+3	T2cNxMx	Positive	Negative
D101606	66	L	3+4	T2aN0Mx	Positive	NA
R103006	57	R	3+4	T2bNxMx	Positive	NA
R103106	76	R, L, A/P, Ap	3+4	T2cNxMx	Positive	NA
D110106	50	R, L, A/P, Ap	4+3+5	T3aN0Mx	Positive	Negative
R110606	58	R, L, A/P, Ap	4+3+5	T2cNxMx	Positive	Negative
B011707	64	R/L, A/P, B	3+3	T2cN0Mx	Positive	Negative
L030207	59	R/L, A/P, B	3+4	T2cN0Mx	Positive	Negative
L030507	62	R/L, A/P	4+5	T3aN0Mx	Positive	Negative

B

C

Prostaspheres form acinar structures in immunocompromised mice

A — Graft	B — UGSM only	C — UGSM + UGSE	D — Spheres only	E — UGSM + Spheres
F	G	H — Control, AR	I — Graft, AR
J — Control, p63	K — Graft, p63	L — CK8	M — CK8
N — CK5	O — CK5	P — PSCA	Q — PSCA
R — PSMA	S — PSMA	T — PSA	U — PSA

SUPPORTING DATA: N/A